Expression of Interleukin-18 in the Lung after Endotoxemia or Hemorrhage-Induced Acute Lung Injury

Patrick G. Arndt, Giamilla Fantuzzi, and Edward Abraham

Divisions of Pulmonary and Critical Care Medicine, and Infectious Diseases, University of Colorado Health Sciences Center, Denver, Colorado

Hemorrhage and endotoxemia are important risk factors for the development of acute lung injury. Interleukin (IL)-18 is a recently described cytokine released in its mature, active form after pro-IL-18 is cleaved by the IL-1 converting enzyme (ICE). IL-18 has multiple immunomodulating properties, including induction of interferon-γ (IFN-γ), IL-1β, tumor necrosis factor-α, and intercellular adhesion molecule-1. To examine the possible involvement of IL-18 in acute lung injury, we examined its expression, as well as that of IFN-γ, IL-12, and ICE, using murine hemorrhage or endotoxemia models. The amounts of IL-18 messenger RNA (mRNA) increased in the lung after hemorrhage or endotoxemia. However, only endotoxemia was associated with elevations in lung and plasma concentrations of IL-18 protein. ICE expression was increased in the lungs after endotoxemia but not after hemorrhage. Although IFN-γ expression increased in the lungs after hemorrhage or endotoxemia, elevations in lung IL-12 mRNA levels were found only after endotoxemia. These results indicate that hemorrhage and endotoxemia induce different patterns of immunomodulatory cytokine expression in the lungs. In particular, differences in the expression of ICE after hemorrhage or endotoxemia may affect generation of the active forms of downstream cytokines, including IL-18. IFN-γ expression in the lungs after hemorrhage appears to occur through a pathway independent of IL-12 and IL-18. IL-18 may play a role in modulating the development of acute lung injury after endotoxemia but not after hemorrhage.

Acute lung injury (ALI) and its most severe form, acute respiratory distress syndrome (ARDS), are important clinical entities affecting approximately 150,000 patients in the United States per year (1, 2). Infection and blood loss are two major, well-described etiologies predisposing to the development of ALI (1, 2). Endotoxemia or hemorrhage induces increases in the expression of proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-1α, interferon (IFN)-γ, and macrophage inflammatory peptide-2 in murine lung cells (3–8), and increased levels of TNF-α and IFN-γ are present in bronchoalveolar lavage fluid (BALF) after hemorrhage (8). These experimental findings correlate with those from clinical studies where elevated levels of immunoregulatory cytokines, including IL-1β, IL-8, TNF-α, and IL-6, are present in the plasma and BALF of patients with ARDS compared with control patients receiving mechanical ventilation (9–12). Recently, the proinflammatory activity of BALF was shown to be significantly greater than that of plasma in patients with ARDS, suggesting that the lung is an active, local site of inflammation in this setting (13, 14).

IL-18, also called interferon gamma inducing factor, is a recently described cytokine initially isolated from hepatic Kupffer cells (15–18). IL-18 is constitutively expressed in lung, skin, skeletal muscle, kidney, and pancreas (18, 19). In *in vitro* studies, IL-18 increases IL-1β, TNF-α, IL-8, IL-2, and granulocyte macrophage colony-stimulating factor (GM-CSF) release (20–22), decreases IL-10 release (20), upregulates intercellular adhesion molecule-1 expression (23), enhances T-cell proliferation (20, 24), increases natural killer cell activity through Fas ligand–mediated pathways (25–27), and can decrease angiogenesis in tumor models (28).

Similar to pro-IL-1β, pro-IL-18 lacks a signal sequence and requires cleavage to its mature and active form, which is then secreted (29, 30). The interleukin-1 converting enzyme (ICE) is the predominant enzyme responsible for the cleavage of pro-IL-18 to mature IL-18 (29–31).

The importance of IL-18 in the underlying pathophysiologic response to endotoxemia, an important risk factor for the development of ALI, was recently illustrated in IL-18 receptor knockout mice that were resistant to the effects of endotoxemia compared with wild-type control mice (27). However, little is known concerning the kinetics of expression of IL-18 in the lungs after endotoxemia or other inflammatory insults, such as blood loss, that are associated with the development of ALI. We therefore investigated the effects of endotoxemia and hemorrhage on IL-18 and ICE expression in the lungs. Because IL-12 and IL-18 have overlapping properties of inducing IFN-γ expression (32–36), we also examined the ability of endotoxemia or hemorrhage to modulate pulmonary expression of IL-12 and IFN-γ.

Materials and Methods

Animals

Male BALB/c mice, ages 8 to 12 wk, were obtained from Harlen Sprague Dawley (Indianapolis, IN) and housed in the University of Colorado Health Sciences Center animal care facility. Mice were kept on a 12-h light/dark cycle with free access to food and water.

Endotoxemia or Hemorrhage Models

The endotoxemia and hemorrhage models used in these experiments have been described previously by our laboratory (3, 4, 6–

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Address correspondence to: Edward Abraham, M.D., Division of Pulmonary and Critical Care Medicine, University of Colorado Health Sciences Center, 4200 East Ninth Avenue, Box C-272, Denver, CO 80262. E-mail: Edward.Abraham@UCHSC.edu

Abbreviations: acute lung injury, ALI; acute respiratory distress syndrome, ARDS; bronchoalveolar lavage fluid, BALF; electrochemiluminescence, ECL; glyceraldehyde-3-phosphate dehydrogenase, G3PDH; hypoxanthine phospho-ribosyl transferase, HPRT; interleukin-1 converting enzyme, ICE; interferon, IFN; interleukin, IL; lipopolysaccharide, LPS; messenger RNA, mRNA; phosphate-buffered saline, PBS; reverse transcriptase/polymerase chain reaction, RT-PCR; standard error of the mean, SEM; tumor necrosis factor, TNF.

8). Both are associated with onset of ALI within 72 h as evidenced by pulmonary neutrophil infiltration, interstitial pulmonary edema, and proteinaceous alveolar exudate (3, 7, 37, 38).

For endotoxemia, mice were injected intraperitoneally with lipopolysaccharide (*Escherichia coli* 0111:B4; Sigma, St. Louis, MO) at the dose of 25 mg/kg in 0.2 ml phosphate-buffered saline (PBS). Control mice received 0.2 ml PBS intraperitoneally. Hemorrhage was performed by removal of 30% of the calculated total blood volume (0.55 ml for a 20-g mouse) by cardiac puncture over 60 s under methoxyflurane anesthesia. With this method, overall mortality is < 12%, with no evidence of bleeding into the pericardial space, hemothorax, or lung or cardiac contusion (3, 4, 6–8). Control animals underwent cardiac puncture under methoxyflurane anesthesia without blood removal.

Lung Isolation and BALF Collection

At predetermined time points, mice were exsanguinated by cardiac puncture under methoxyflurane anesthesia. The blood was drawn into a heparinized syringe (5 U heparin), then centrifuged at 2,500 rpm for 10 min for plasma collection. After death, the chest cavity was opened, the right ventricle flushed with 3 to 5 ml of PBS at 4°C, and the lungs removed with avoidance of the peritracheal lymph nodes (3, 5, 8). The lungs were then snap-frozen in liquid nitrogen with the right lung used for RNA extraction and the left lung used for protein analysis.

Semiquantitative Reverse Transcriptase/Polymerase Chain Reaction

The techniques used for semiquantitative reverse transcriptase/polymerase chain reaction (RT-PCR) have been previously described by our laboratory (3–8). Total RNA was isolated from the right lung by phenol-chloroform extraction after homogenization in 2 ml of 4 M guanidine thiocyanate/5 mM sodium citrate/0.5% sarcosyl and 0.1 M 2-mercaptoethanol. Complementary DNA (cDNA) was then synthesized from 1 μg total RNA using Maloney murine leukemia virus reverse transcriptase and random hexamer oligonucleotides as previously described by our laboratory (3, 4, 6–8).

Semiquantitative PCR for IL-18, IFN-γ, IL-12, and ICE was performed using 5 μl of the cDNA mixture under the following cycle conditions (except for ICE): initial 2 min 94°C denaturation step followed by 30 to 38 cycles of 60 s, 94°C denaturation; 60 s, 55 to 65°C annealing (depending on cytokine primers); and 60 s, 72°C extension. A final 4-min extension at 72°C was then performed. For ICE, the cycle conditions were an initial 1 min 95°C denaturation step followed by 38 to 40 cycles of 30 s, 94°C denaturation; 30 s, 54°C annealing; and 60 s, 72°C extension. This was followed by a 5-min 72°C final extension step. PCR products were visualized by electrophoresis on 1.6% agarose gels with ethidium bromide staining. Cycle number was adjusted so that the PCR products were between barely visible and below saturation. The housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and hypoxanthine phospho-ribosyl transferase (HPRT) were used as controls. Analysis of the gel was done by densitometry (ImageStore 5000; UVP, San Gabriel, CA) with relative absorbance determined by comparison of the density of the PCR product of the cytokine of interest to the housekeeping gene.

Measurement of IL-18 Protein

For assessment of IL-18 protein levels, an electrochemiluminescence (ECL) assay was used. The techniques used for the ECL assay have been described previously (22). Briefly, the left lung was homogenized in lysis buffer containing *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid 10 mM, NaCl 150 mM, Igepal 1% vol/vol, ethylenediaminetetraacetic acid 1 mM, leupeptin 1 μg/ml, phenylmethylsulfonyl fluoride 100 μg/ml, aprotinin 1 μg/ml, pepstatin 1 μg/ml, and soybean trypsin inhibitor 1 μg/ml; centrifuged at 2,500 rpm for 10 min; and the supernatant was then removed, aliquoted, and kept at −80°C until analyzed for IL-18 levels. Antibodies used were an affinity-purified, antimurine IL-18 polyclonal antibody (R&D Systems, Minneapolis, MN) that was labeled with bioten (Igen Inc., Gaithersburg, MD) as per manufacturer's protocol, and a monoclonal, antimurine IL-18 antibody (R&D Systems) labeled with ruthenium (II) trisbipyride chelate (Igen) as per the manufacturer's instructions. The biotinylated antibody was diluted to a final concentration of 0.5 μg/ml in PBS (pH 7.4) containing 0.25% bovine serum albumin, 0.5% Tween-20, and 0.01% azide (ECL buffer). In each assay tube, 25 μl of the above biotinylated anti-IL-18 antibody was incubated at room temperature with 25 μl of a 1-mg/ml solution of streptavidin-coated paramagnetic beads (Dynal Corp., Lake Success, NY) diluted in ECL buffer for 30 min with mixing by vigorous shaking. To each assay tube, 25 μl of sample or standard concentrations of recombinant murine IL-18 (Pepro-Tech Inc., Rocky Hill, NJ) were added, followed by 25 μl of the ruthenylated antibody (final concentration 1 mg/ml, diluted in ECL buffer), and incubated overnight at room temperature with shaking. The reaction was quenched with 200 μl of PBS per tube and the amount of chemiluminescence was determined using an Origen 1.5 analyzer (Igen Inc.). A standard curve was constructed using recombinant IL-18 (Pepro-Tech, Inc.). The IL-18 ECL detects both pro- and mature IL-18 with a sensitivity of 20 pg/ml.

Statistical Analysis

Groups of 5 to 8 mice were used for each time point. For each experimental condition, groups of control, unmanipulated animals were included. Data are presented as mean normalized to controls ± standard error of the mean (SEM) for each experimental group. Groups were compared using one-way analysis of variance and the Tukey-Kramer multiple comparison tests for differences between groups. A P value < 0.05 was considered to be statistically significant.

Results

IL-18 Expression Is Increased in the Lung after Endotoxemia or Hemorrhage

As reported previously (18, 19), we found that IL-18 messenger RNA (mRNA) is constitutively expressed in the lung (Figure 1). As early as 1 h after endotoxemia, there was increased expression of IL-18 mRNA in the lungs, followed by a decrease to below baseline levels of expression 4 h after endotoxin administration. No detectable IL-18 mRNA, even with 40 cycles of PCR, could be found 8 h after endotoxemia (Figures 1A and 1B).

Amounts of IL-18 mRNA were increased in the lung after hemorrhage. As shown in Figure 1, IL-18 expression in the lungs increased more slowly after hemorrhage than after endotoxemia, with peak in IL-18 mRNA levels being present 4 h after hemorrhage with return to below baseline levels 4 h later.

IL-18 Protein Levels Are Increased in the Lung and Plasma after Endotoxemia but Not after Hemorrhage

Because alteration in IL-18 mRNA levels may not necessarily correlate with changes in protein, we investigated IL-18 protein in the lung and plasma of mice either given endotoxin or hemorrhaged. Significant increases in IL-18 protein were present in the lungs 1 h after endotoxemia (Figure

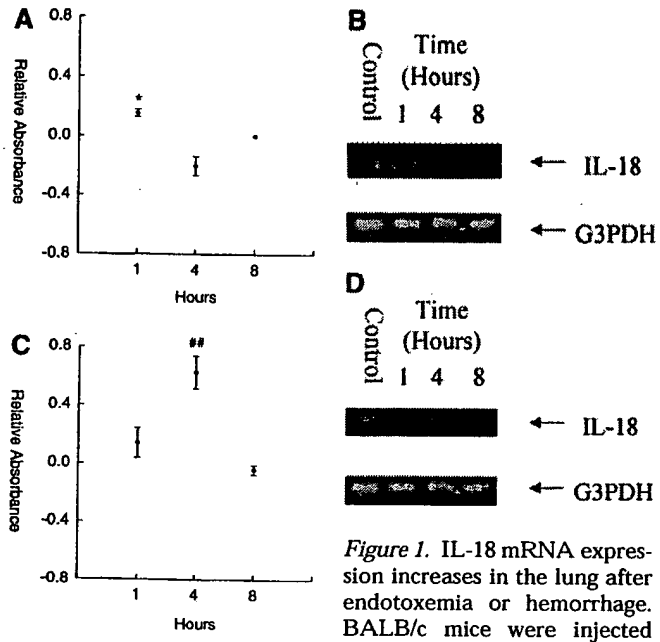

Figure 1. IL-18 mRNA expression increases in the lung after endotoxemia or hemorrhage. BALB/c mice were injected with LPS or PBS as control (*A* and *B*) or were hemorrhaged 30% of their blood volume (*C* and *D*). At the indicated time points, RT-PCR for IL-18 was performed. No IL-18 PCR product was visualized 8 h after endotoxemia, even after 40 cycles. Results are normalized to G3PDH and are expressed as relative absorbance normalized to controls ± SEM. $^{*}P < 0.05$, $^{\#}P < 0.001$. Representative gels for IL-18 mRNA expression after LPS (*B*) or hemorrhage (*D*) are shown.

2A). Pulmonary IL-18 levels remained significantly elevated, compared with those present in unmanipulated mice, 4 h after endotoxemia, and then returned to baseline levels 8 h after endotoxin administration. In contrast to the increased levels of IL-18 present in the lung after endotoxemia, we did not find any significant alterations in IL-18 protein in the lung during the 8-h period after hemorrhage. In bronchoalveolar lavage, IL-18 was undetectable in control, unmanipulated mice and at all time points after endotoxemia or hemorrhage (data not shown).

In plasma, low levels of IL-18 were found at baseline. Increases in plasma IL-18 occurred as early as 1 h after endotoxin administration, reached statistical significance 4 h after endotoxemia, and then returned to baseline 4 h later (Figure 2B). In contrast to endotoxemia, hemorrhage did not produce any statistically significant changes in plasma IL-18 levels (data not shown).

IFN-γ Expression Is Increased in the Lung after Endotoxemia or Hemorrhage

IFN-γ mRNA levels rose after endotoxin injection, with significant increases, compared with baseline, being present 4 and 8 h after endotoxin administration (Figure 3A). The expression of IFN-γ mRNA in the lung also increased after hemorrhage, but with kinetics different from those seen after endotoxin administration. The peak in expression of IFN-γ mRNA occurred 4 h after hemorrhage and was of short duration, with a return to below baseline 8 h after blood loss (Figure 3B).

Expression of IL-12 mRNA Is Increased in the Lung after Endotoxemia but Not after Hemorrhage

IL-12 induces IFN-γ expression both alone and in synergy with IL-18, in part by upregulating IL-18 receptors (32–36). In the present experiments, there was a rapid, but transient, increase in the expression of IL-12 mRNA in the lung after endotoxin administration (Figure 4). IL-12 mRNA levels were significantly greater than those present in the lungs of control, unmanipulated mice 1 h after endotoxin administration. By 4 h after endotoxin administration, IL-12 expression was similar to baseline. After hemorrhage, the only significant alteration in IL-12 expression was a decrease 8 h after blood loss.

ICE mRNA Expression Is Increased in the Lung after Endotoxemia but Not after Hemorrhage

Because ICE is important in cleaving pro-IL-18 to mature IL-18 (29–31), we investigated its expression in the lung after endotoxemia or hemorrhage. ICE mRNA was constitutively present in the lung (Figure 5), and then increased 4 h after endotoxemia, before returning to baseline levels 8 h after endotoxin administration (Figure 5). After hemorrhage, there was no alteration in the expression of ICE mRNA in the lung at any of the time points investigated.

Discussion

In these experiments, we found that endotoxemia and hemorrhage produce rapid increases in the expression of IL-18 mRNA in the lung, present between 1 and 4 h, re-

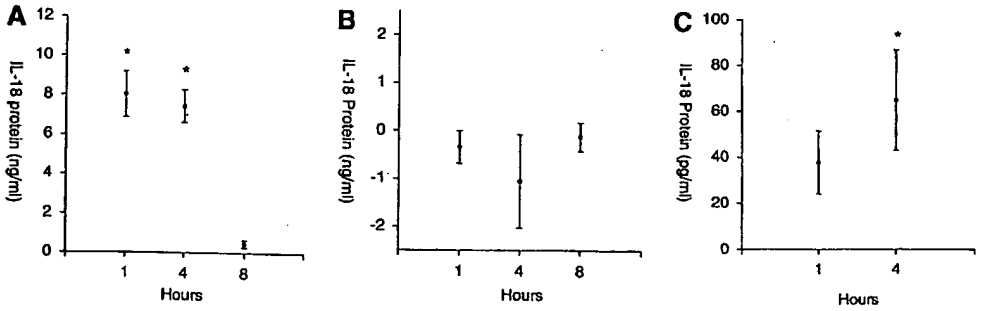

Figure 2. (*A* and *B*) IL-18 protein levels are increased in the lung after endotoxemia, but not after hemorrhage. BALB/c mice were injected with LPS or PBS as control (*A*) or were hemorrhaged 30% of their total blood volume (*B*). At the indicated time points, whole lung IL-18 protein was determined. Results are normalized to controls and are expressed as mean ± SEM. $^{*}P < 0.001$. (*C*) IL-18 protein levels are increased in plasma after endotoxemia, but not after hemorrhage. BALB/c mice were injected with LPS or PBS as control. At the indicated time points, plasma was collected and IL-18 levels determined. Results are normalized to controls and are expressed as mean ± SEM. $^{*}P < 0.05$.

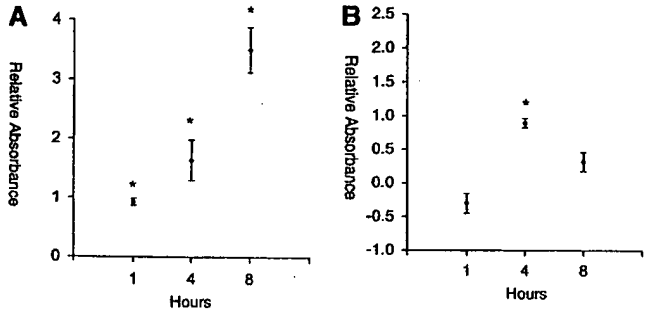

Figure 3. IFN-γ mRNA expression increases in the lung after endotoxemia or hemorrhage. BALB/c mice were injected with LPS or PBS as control (*A*) or were hemorrhaged 30% of their total blood volume (*B*). At the indicated time points, RT-PCR for IFN-γ was performed. Results are normalized to G3PDH and are expressed as relative absorbance normalized to controls ± SEM. $*P < 0.001$.

spectively, after each of these conditions. The kinetics of IL-18 mRNA expression in the lung after endotoxemia or hemorrhage are consistent with those reported in previous *in vitro* studies (39, 40). In keratinocytes exposed to contact allergens, IL-18 mRNA levels increased between 4 and 6 h (39). When human peripheral blood mononuclear cells are exposed to endotoxin, IL-18 mRNA levels peak between 2 and 6 h (40). In the only other *in vivo* study investigating IL-18 after endotoxemia, an increase in IL-18 mRNA levels in murine splenocytes was found within 2 h after endotoxin administration (41). In that study, pulmonary expression of IL-18 was not examined (41).

In addition to inducing increases in IL-18 mRNA expression, we found that endotoxin administration was followed by increased IL-18 protein in the lung and plasma. The elevations in lung and plasma IL-18 protein levels were of short duration, occurring between 1 and 4 h after endotoxemia, and suggest that IL-18 may be active in the lung early in the proinflammatory response. Our findings of increased IL-18 plasma levels after endotoxemia are similar to the kinetics of IL-18 protein levels seen in an earlier *in vivo* study (15) where IL-18 serum levels peaked 2 h after endotoxemia, with a gradual return to baseline over the next 4 h.

We did not find an increase in IL-18 protein levels in the lung or plasma after hemorrhage, suggesting that there are differences in the post-transcriptional regulation and therefore probable importance of IL-18 in these two models of ALI. The explanation for the lack of an increase in IL-18 levels after hemorrhage, in the face of elevated IL-18 mRNA expression, may be related to differences in ICE activity after endotoxemia or hemorrhage. We found that ICE expression in the lung was increased after endotoxemia, but not after hemorrhage. Unlike pro-IL-1β where proteases other than ICE are able to generate mature IL-1β, ICE is currently the only known protease able to cleave pro-IL-1β into its mature and active form (29–31).

Endotoxin-associated increases in IFN-γ mRNA levels occurred after the peak in IL-18 mRNA expression and simultaneously with the peak in IL-18 plasma levels. This suggests that IL-18 may be responsible for the enhanced expression of IFN-γ in the lung after endotoxemia. Previous studies showed that IL-18 occupies an important role in inducing IFN-γ expression after endotoxemia (15, 16, 27, 31). In particular, IFN-γ expression was markedly reduced both in ICE and in IL-18 knockout mice compared with wild-type control mice after endotoxin administration (27, 31).

Because IL-18 protein levels do not increase in the lungs after hemorrhage, other factors would appear to be responsible for inducing IFN-γ expression in this setting. The importance of IL-18 in inducing IFN-γ expression af-

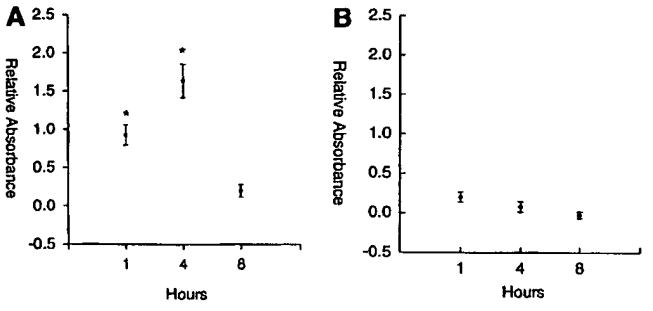

Figure 4. IL-12 mRNA expression is increased in the lung after endotoxemia, but not after hemorrhage. BALB/c mice were injected with LPS or PBS as control (*A*) or were hemorrhaged 30% of their total blood volume (*B*). At the indicated time points, RT-PCR for IL-12 was performed on excised lungs. Results are normalized to HPRT and are expressed as relative absorbance normalized to controls ± SEM. $*P < 0.001$.

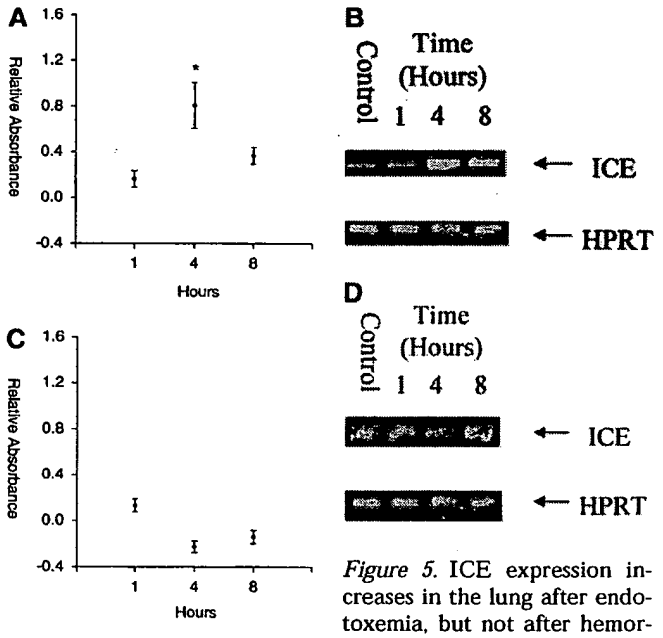

Figure 5. ICE expression increases in the lung after endotoxemia, but not after hemorrhage. BALB/c mice were injected with LPS or PBS as control (*A* and *B*) or were hemorrhaged 30% of their total blood volume (*C* and *D*). At the indicated time points, RT-PCR for ICE was performed. Results are normalized to HPRT and are expressed as relative absorbance normalized to controls ± SEM. $*P < 0.001$. Representative gels for ICE mRNA expression after LPS (*B*) or hemorrhage (*D*) are shown.

ter inflammatory insults other than endotoxemia has not been well characterized. A recent study showed that IL-18 is important for IFN-γ expression after zymosan exposure (31). However, there are no previous studies that have investigated interrelationships between IL-18 and IFN-γ after hemorrhage.

IL-2, TNF-α, and IL-12 can increase IFN-γ expression (15, 33–36, 42). IL-12, although considered a weaker inducer of IFN-γ than IL-18, increases IFN-γ expression, both primarily and in synergy with IL-18 (32–36). In our study, we failed to find an increase in IL-12 mRNA levels in the lung after hemorrhage, indicating that IL-12 was unlikely to have been responsible for the observed effects on IFN-γ. In previous studies (4), we found that hemorrhage resulted in upregulation of TNF-α, but not of IL-2, in the lungs, suggesting that TNF-α, but not IL-2, may play a role in modulating IFN-γ expression after blood loss. Additionally, signaling through the p38 mitogen-activated protein (MAP) kinase pathway has previously been shown to upregulate IFN-γ expression *in vitro* (43). This suggests that other mediators, such as IL-1β, IL-3, and GM-CSF, which use the p38 MAP kinase signaling pathway, may be involved in the induction of IFN-γ expression in the lung after hemorrhage (43).

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